Gene Expression Profiling of Hyperkeratotic Skin From Inner Mongolians Chronically Exposed to Arsenic

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INTRODUCTION

Chronic arsenic exposure has been correlated with the development of several human cancers including those found in the lung, skin, liver, kidney and urinary bladder. Most arsenic exposure in humans is related to the consumption of contaminated drinking water, and millions of people worldwide are exposed to drinking water concentrations that greatly exceed the current World Health Organization's recommended limit of 10 ppb.

The first clinical signs of chronic arsenic exposure are often found in the skin, a major target organ of arsenic toxicity. These include nonmalignant lesions such as hyperkeratoses and areas of hyper/hypopigmentation. More serious skin diseases, such as Bowen's Disease (squamous cell carcinoma *in situ*) and non-melanoma skin cancers may develop over time. The hyperkeratotic lesions can be precursors of arsenic-related skin cancers, but the mechanisms of their conversion to malignancies (and mechanisms of arsenic carcinogenesis in general) are not well understood.

Arsenic-related hyperkeratoses.

OBJECTIVES

To better understand the mechanisms of arsenic carcinogenesis, we performed global gene expression profiling on RNA obtained from hyperkeratotic skin lesions from individuals suffering from chronic arsenic exposure. These individuals are from arsenic endemic areas in Inner Mongolia, and had been exposed to high levels of inorganic arsenic (212-950 ppb) in their drinking water for ≥20 years. These transcriptional profiles were compared to those obtained from RNA isolated from unlesioned skin from individuals in a nearby area with low lifetime exposures to arsenic in their drinking water (~7 ppb).
This is the first gene expression study involving a comprehensive microarray (54,675 probe sets) that utilizes arsenic-related lesions from the skin, a major target organ of arsenic toxicity.

MATERIALS & METHODS

Study Subjects and Skin Sample Collection

This study was conducted according to the recommendations of the World Medical Association Declaration of Helsinki for international health research. The research protocol met the requirements for protection of human subject certification as approved by the U.S. Environmental Protection Agency. All subjects gave written, informed consent to participate in this study and completed questionnaires that provided information regarding sociodemographic characteristics, tobacco use, medical history and arsenic exposure information (Table 1). A total of 11 study subjects were recruited: 7 individuals from the arsenism endemic areas of Zhi Ji Liang and Bayingnormen ('Ba Men') in Inner Mongolia and 4 individuals from Huhhot (the capital of Inner Mongolia). The 7 Ba Men/Zhi Ji Liang individuals had skin hyperkeratoses consistent with chronic arsenism and been exposed to 212-950 ppb arsenic in their drinking water for ≥20 years. Skin hyperkeratoses were diagnosed by a dermatologist according to the China National Standards for Diagnosis of Arsenicosis of The People's Republic of China. The 4 Huhhot individuals had lifetime arsenic exposures of ~7 ppb in their drinking water and exhibited no symptoms of arsenism. A skin sample from each study subject (hyperkeratotic lesions from Ba Men/Zhi Ji Liang individuals; unlesioned skin from Huhhot individuals) was collected by a dermatologist and immediately placed in RNAlater® (Ambion, Austin, Texas) to stabilize the RNA in the samples. The samples were transported on ice packs via air to the United States and stored at -20° C.

RNA Extraction

For total RNA isolation, approximately half of each ~0.2 cm X 0.3 cm skin sample, including epidermis and some dermal tissue, was pulverized using a mortar and pestle under liquid nitrogen. Immediately after the liquid nitrogen had evaporated, 1 ml of buffer RLT from the RNeasy Mini Kit (Qiagen, Valencia, CA) was added to the sample. The sample was transferred to a tube and further homogenized for 45 seconds in an electric homogenizer at low speed (Tissue Tearor model 985-370, Biospec Products, USA). Subsequently, the RNeasy Mini Kit (Qiagen) protocol was used to purify total RNA, yielding 225 ng-8,520 µg of total RNA per sample. RNA concentrations and quality were determined spectrophotometrically using A_{260} and A_{260}/A_{280}, respectively. RNA quality was further verified using the RNA 6000 Nano Assay and the Bioanalyzer 2100 (Agilent, Palo Alto, CA). Only RNA samples of good quality were used in the study (A_{260}/A_{280} of 1.9-2.1 and the presence of two distinct, intact 18S and 28S ribosomal RNA populations identified using the Bioanalyzer 2100). RNA samples of good quality were obtained from the 4 unlesioned skin samples from healthy individuals (control samples) and hyperkeratotic (HK) lesions from arsenism subjects (HK samples).

Microarray analyses

Total RNA from each sample (4 control and 7 HK samples) was used for global gene expression profiling performed by Expression Analysis, Inc. (Durham, NC). Biotinylated cRNA was obtained from 50 ng of total RNA from each sample using Affymetrix® Two-Cycle Target Labeling Assay and was hybridized to GeneChip® Human U133 Plus 2.0 Arrays (containing 54,675 probe sets) according to the manufacturer's instructions (Affymetrix®., Santa Clara, CA). The output files (.cel files) were subsequently analyzed for differential gene expression between control and HK samples using the methods described by Choe et al. (2005) using a t-test ($p \leq 0.05$) with a Benjamini and Hochberg multiple testing correction. Pathway and functional analyses of the differentially expressed genes were performed using Genespring® (v. 7.2), DAVID 2.0 (NIAID), BioCarta, Ingenuity Pathways Analysis, GeneGo and Bio Resource for Array Genes (BioRag). Principle Components Analyses were performed using Cluster 3.0..

Determination of arsenic concentration in drinking water

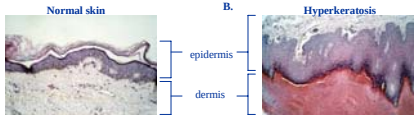
Water samples collected from the subjects' homes were analyzed for arsenic content using hydride generation atomic fluorescence spectrometry (HGAFS) (Le and Ma, 1998) or a standard colorimetric method using silver diethyldithiocarbamate (Zhang et al., 1994).

Table 1. Description of donors and skin samples used in gene expression profiling.

Donor/skin sample number	Donor sex	Donor age (years)	Smoking status	[As] in drinking water (ppb)	Skin sample location	Skin sample diagnosis
C-5	M	33	Yes	7	abdomen	normal
C-6	M	35	Yes	7	arm	normal
C-7	M	41	No	7	arm	normal
C-8	F	37	No	7	arm	normal
332	F	42	No	671	hand	hyperkeratosis
333	M	31	No	950	hand	hyperkeratosis
334	M	48	Yes	480	foot	hyperkeratosis
336	F	33	No	212	foot	hyperkeratosis
338	M	26	Yes	940	hand	hyperkeratosis
342	F	61	Yes	212	hand	hyperkeratosis
343	M	37	Yes	610	foot	hyperkeratosis

RESULTS

PATHOLOGY

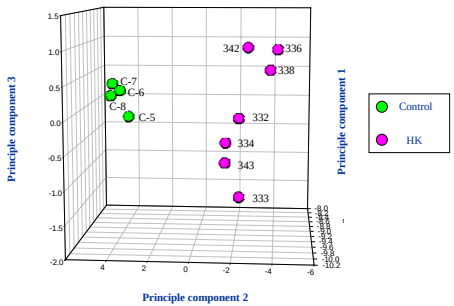


Representative hematoxylin and eosin stained sections from normal and hyperkeratotic skin samples used in the study. (A) Normal skin from abdomen of subject C-5 (4X). (B). Lesioned skin from hand of subject 332, showing hyperkeratosis and acanthosis (10X).

GENE EXPRESSION SUMMARY

- In all samples, ~39% of the 54,675 probe sets present on the Human U133 Plus 2.0 arrays were expressed.
- Of the ~21,300 expressed probe sets, 2824 were identified by statistical analyses to be differentially expressed between unlesioned (control) and hyperkeratotic (HK) skin samples.

PRINCIPLE COMPONENTS ANALYSIS



Principle components analysis (PCA) demonstrating separation of control and HK sample groups was performed using Gene Cluster 3.0 and the 2824 differentially expressed probe sets. Samples are labeled according to the donor sample numbers described in Table 1.

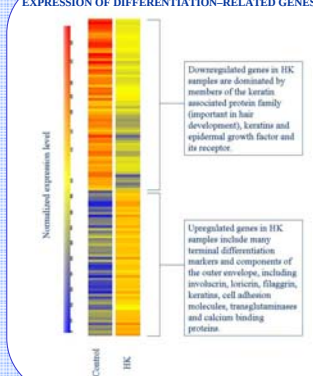
SIGNIFICANT ALTERED PATHWAYS

The most significant metabolic and cellular and regulatory pathways represented in the differentially expressed probe sets are listed as determined by BioRag ($p \leq 0.001$). Pathways are listed in order of decreasing statistical significance (incomplete lists).

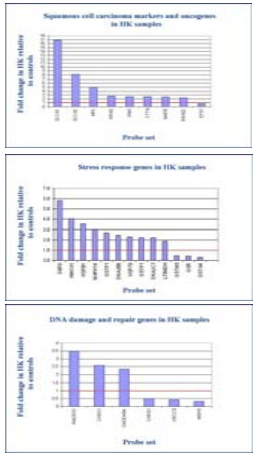
BioCarta pathways	GenMAPP pathways	KEGG pathways
Keratinocyte differentiation	Apoptosis	Xenobiotic metabolism by p450
EGF signaling	Glycolysis and gluconeogenesis	MAPK signaling
Erk/Erk2 MAPK signaling	TGF beta signaling	Regulation of actin cytoskeleton
p38 MAPK signaling	Wnt signaling	Cell cycle
Wnt signaling	Cell cycle	Cell adhesion molecules
Stress Induction of HSP regulation		Gap junction
Cell cycle: G1/S checkpoint		Cell communication
p53 signaling		Apoptosis
Cell cycle: G2/M checkpoint		
Oxidative stress induced gene expression via Nrf2		
Ras signaling		

One of the most dominant and significant features of the differentially expressed probe sets was the modulation of genes involved in epidermal development and differentiation. The distinct keratin gene expression profiles indicated (*) are consistent with those found in the activated epidermis, hyperproliferative skin diseases, and immunohistochemical studies of arsenic-related skin lesions described in the literature.

EXPRESSION OF DIFFERENTIATION–RELATED GENES



SELECT GENE GROUPS OF INTEREST IN HK SAMPLES



CONCLUSIONS/FUTURE STUDIES

Gene expression changes in arsenic-exposed HK lesions are consistent with many of the proposed mechanisms of arsenic carcinogenesis, including induction of oxidative stress, disruption of DNA repair, and modulation of apoptosis, cell proliferation and cell differentiation. Oncogenes and squamous cell carcinoma biomarkers are also upregulated in these lesions. Detailed pathways connecting these processes are being constructed and may give more insight into the important events in arsenic carcinogenesis. These expression profiles are also being compared to normal human epidermal keratinocytes exposed to environmentally-relevant concentrations of arsenic *in vitro* and will be confirmed by quantitative real-time PCR.

REFERENCES

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